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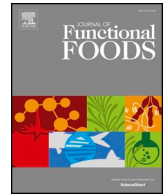
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Human milk oligosaccharides and its acid hydrolysate LNT2 show immunomodulatory effects via TLRs in a dose and structure-dependent way

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ABSTRACT

Human milk oligosaccharides (HMOs) have beneficial immune effects but the mechanisms of action are not well-understood. Here we study Toll-like receptor (TLR) signaling of the HMOs 2'-FL, 3-FL, 6'-SL, LNT2, and LNnT and their effect on cytokine production in human macrophages. 3-FL activated TLR2 and LNT2 activated all TLRs in a dose-dependent way. In an inhibition assay, 2'-FL, 6'-SL, and LNnT inhibited TLR5 and 7, while 3-FL inhibited TLR5, 7, and 8. 6'-SL showed a synergistic effect on ssRNA40-induced TLR8 activation. In addition, we measured HMO-induced cytokine production in THP1 macrophages. IL-10 and TNF α were induced by LNT2, and the effects were NF- κ B dependent, while the other HMOs had minor effects. The potent effects of LNT2 might be explained by the unique N-acetylglucosamine end that binds to leucine-rich repeats on TLRs. Our data suggest that the effects of HMOs on TLR signaling and immunomodulation of macrophages are HMO-structure dependent.

1. Introduction

Breastfeeding is the golden standard for infant feeding in the first 6 months of life (Walker, 2010). It provides the necessary nutrition to the infant but also supports the immune defense in the phase of the immaturity of the immune system of the neonates. It has been shown for example that breastfed infants have a lower risk of infection and inflammation than formula-fed babies (Klement, Cohen, Boxman, Joseph, & Reif, 2004; Ladoomenou, Moschandreas, Kafatos, Tselentis, & Galanakis, 2010). Human milk oligosaccharides (HMOs) have been shown to be one of the responsible molecules in human milk for the immune supporting effects in neonates (Buescher, 2001).

HMOs are non-digestible carbohydrates comprising a family of more than 200 different oligosaccharides with highly diverse structures (Bode, 2012; Kunz, Rudloff, Baie, Klein, & St, 2000). HMOs can be found in mother's milk in concentrations varying between 5 and 20 mg/mL (Bode, 2012; Petherick, 2010), depending on the stage of lactation

and may provide a variety of health-promoting effects (Bode, 2012; Kunz et al., 2000; Smilowitz, Lebrilla, Mills, German, & Freeman, 2014). These health effects vary from promoting gut microbiota development (Asakuma et al., 2011), reducing pathogenic infections by acting as anti-adhesive molecule (Morrow et al., 2004), supporting brain development and cognition (Belfort, 2018; Wang, 2009), but also playing a role in supporting development of the mucosal and systemic immune system (Ayechu-Muruzabal et al., 2018; He, Liu, Leone, & Newburg, 2014). Which HMO is responsible for which health effect is not completely known but subject of intensive research efforts.

HMOs have been shown to stimulate immune function in different ways (Bode et al., 2004; Lane, O'Callaghan, Carrington, & Hickey, 2013; Velupillai & Harn, 1994; Vos et al., 2007). For example, colostrum derived HMOs attenuated pathogen-associated molecular pattern (PAMP) induced acute phase inflammatory cytokine production in intact immature human intestinal mucosa and the immature intestinal epithelial cell line H4 (He et al., 2014). Simultaneously, it stimulated

Abbreviations: 2'-FL, 2'-fucosyllactose; 3-FL, 3-fucosyllactose; 6'-SL, 6'-sialyllactose; DC, dendritic cell; DSLNT, Disialyllacto-N-tetraose; ECDs, N-terminal ectodomains; HEK, Human Embryonic Kidney; HMOs, Human milk oligosaccharides; LAL, Limulus Amebocyte Lysate; LNFP I, Lacto-N-fucopentaose I; LNFP III, Lacto-N-fucopentaose III; LNT2, Lacto-N-Triaose; LNnT, Lacto-N-neotetraose; LRR, Leucine-rich repeats; NF- κ B, Nuclear factor kappa-light-chain-enhancer of activated B cells; PAMP, Pathogen-associated molecular pattern; PMA, Phorbol 12-myristate 13-acetate; SEAP, Secreted Embryonic Alkaline Phosphatase; THP1, Human monocytic cell; Th, T helper cell; TLRs, Toll-like receptors; TNF α , Tumor Necrosis Factor alpha

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tissue repair and homeostasis (He et al., 2014). The HMO 2'-fucosyllactose (2'-FL) attenuated interleukin (IL)-8 production which was induced by lipopolysaccharide (LPS) derived from type 1 pili pathogens (He, Liu, et al., 2016). Disialyllacto-N-tetraose (DSLNT) also have been identified as immunomodulating and immune attenuating HMO. It suppresses necrotising enterocolitis-like inflammation in neonatal rats (Jantscher-Krenn et al., 2012). However, there are also HMOs that have immune stimulating properties. The HMO 3'-sialyllactose (3'-SL), for example, showed immune stimulating characteristics by stimulating mesenteric lymph node CD11c⁺ dendritic cells to increase the production of IFN- γ , TNF- α , IL-12, and TGF- β 1 that induced Th1 and Th17 cell frequencies (Kurakevich, Hennot, Hausmann, Rogler, & Borsig, 2013). Also, the HMO lacto-N-fucopentaose III (LNFP III) was found to have immunomodulatory activities that induced strong Th2 responses and promoted dendritic cell 2 (DC2) maturation (Thomas et al., 2014).

Some studies suggest that immune effects by HMOs can be induced via Toll-like receptors (TLRs) (He, Lawlor, & Newburg, 2016). TLRs are a family of pattern recognition receptors (PRRs) that play an important role in immune signaling, and TLR signaling is central to innate immunity (He, Lawlor, et al., 2016; Kawai & Akira, 2010). TLRs are expressed by many cell types, including by most immune cells and epithelial cells (Abreu, 2010; Kawai & Akira, 2010). Their ligands are PAMPs but also multiple dietary molecules can be recognized by TLRs (Figuerola-Lozano, Valk-Weeber, van Leeuwen, Dijkhuizen, & de Vos, 2018; Kiewiet et al., 2017; Sahasrabudhe et al., 2018; Vogt et al., 2013). Activation of TLRs might lead to immune signaling through NF- κ B resulting in the modulation of cytokine release (Kawai & Akira, 2010; Medzhitov, 2001). A study using a human colonic cell line (HT-29) showed that lacto-N-fucopentaose I (LNFP I) increased the gene expression of TLR4, while 3'-sialyllactose (3'-SL), 6'-sialyllactose (6'-SL) or 6'-galactosyllactose (6'-GL) enhanced both TLR2 and TLR4 expression (Asakuma et al., 2010). This study also showed that TLR4 not only mediates the effect of LNFP III on promoting DC2 maturation (Thomas et al., 2014), but also the DC response induced by 3'-SL (Kurakevich et al., 2013). A study on H4 intestinal cells showed that 3'-, 4'-, and 6'-GL attenuated the polyinosinic:polycytidylic acid induced inflammatory response through TLR3 (He et al., 2014).

Although some studies have demonstrated that HMOs have the ability to modulate immunity via TLR signaling, the relative effects of individual oligosaccharides on TLRs are still not well-understood. HMOs are also subject to modifications during passage through the gastrointestinal tract. Research shows that acidic HMO fractions among are hydrolyzed at low pH (Gnoth, Kunz, Kinne-Saffran, & Rudloff, 2000). This may lead to production of products such as lacto-N-triose (LNT2), which is the acid hydrolysates of the tetra and higher HMOs such as lacto-N-tetraose (LNT) and lacto-N-neotetraose (LNnT) (Bidart, Rodríguez-Díaz, & Yebra, 2016; Deo & Park, 2006; Gnoth et al., 2000). Therefore, in the present study, we compared the effects of different HMOs (2'-FL, 3-FL, 6'-SL and LNnT) and its acid hydrolysate LNT2 on TLR signaling. The activation and inhibition effects on TLR 2, 3, 4, 5, 7, 8, and 9 and the immunomodulatory effects on human THP1 macrophages were assessed.

2. Materials and methods

2.1. HMOs

In the present study, five different human milk oligosaccharides (HMOs) 2'-FL (provided by FrieslandCampina Domo, Amersfoort, the Netherlands), 3-FL, 6'-SL, LNT2, and LNnT (provided by Glycosyn LLC, Woburn, MA, USA) were tested. An overview of the structure and components of the selected HMOs are shown in Table 1. All samples were tested after 0.2 μ m filtration for endotoxins by using Limulus Amebocyte Lysate (LAL) Chromogenic Endotoxin Quantitation Kit (Pierce™ LAL Chromogenic Endotoxin Quantitation Kit, Thermo Scientific, Breda, The Netherlands) according to the manufacturer's

Table 1

Overview of the structure of selected HMOs and its acid hydrolysate LNT2.

Name (abbreviated)	Structure	Schematic diagram
2'-FL	Fuc α 1-2Gal β 1-4Glc	
3-FL	Gal β 1-4Glc Fuc α 1-3/	
6'-SL	NeuNAc α 2-6Gal β 1-4Glc	
LNT2	GlcNAc β 1-3Gal β 1-4Glc	
LNnT	Gal β 1-4GlcNAc β 1-3Gal β 1-4Glc	

instructions (Fig. S1).

2.2. Cell culture of THP1 and HEK reporter cell lines

To study the effects of HMOs on TLR signaling, the human acute monocytic leukemia reporter cell line (THP1) (InvivoGen, Toulouse, France) and 7 human embryonic kidney (HEK) reporter cell lines (HEK-Blue™-hTLRX) (InvivoGen, Toulouse, France) were used. The THP1 cells line (THP1-XBlue™-MD2-CD14) expresses TLRs endogenously and carries an insert for MD2 and CD14 which boosts TLR signaling. HEK-Blue reporter cell lines express a construct for individual TLRs (TLR2, 3, 4, 5, 7, 8, and 9). Both the THP1 and HEK reporter cell lines also contain a Secreted Embryonic Alkaline Phosphatase (SEAP) construct, which is coupled to the nuclear factor κ B/Activating protein-1 (NF- κ B/AP-1) promoter. In all these cells, activation of TLRs leads the expression of the SEAP gene. The SEAP secretion in the medium can, therefore, be assessed as a measure for the induced TLR activation (Chanput, Mes, & Wichers, 2014).

THP1 cells were passaged twice a week by inoculating 5×10^5 cells. Cells were cultured in RPMI 1640 medium (Gibco, Life Technologies, Bleiswijk, The Netherlands), containing 10% heat-inactivated FBS (Fetal Bovine Serum, HyClone, Thermo Scientific, Breda, The Netherlands), 2 mM L-glutamine, 1.5 g/L sodium bicarbonate (Boom B.V. Meppel, The Netherlands), 4.5 g/L glucose, 10 mM HEPES, 1.0 mM sodium pyruvate, 100 mg/mL Normocin™ (Invivogen, Toulouse, France), and 50 U/mL and 50 μ g/mL Penicillin/Streptomycin. All additives were purchased from Sigma Aldrich (Zwijndrecht) unless indicated otherwise.

HEK-Blue cells were maintained in Dulbecco's modified eagle's medium (DMEM) (Gibco, Life Technologies, Bleiswijk, The Netherlands) with 10% heat-inactivated FBS, 2 mM L-glutamine, 4.5 g/L glucose, 50 U/mL and 50 mg/mL penicillin/streptomycin, and 100 mg/mL Normocin. HEK cells were grown to approximately 80% confluency.

All the reporter cell lines were passaged three times before they were maintained in their respective selection medium according to the manufacturer's protocol.

2.3. THP1 reporter cell stimulation and Quanti-Blue assay

THP1 cells were centrifuged for 5 min at 300g, and cells were re-suspended in culture medium, using the cell density per well as indicated in Table 2. Next, cells were seeded in a flat bottom 96 wells plate (100 μ L). Cells were preincubated with or without 50 μ M MyD88 inhibitor Pepinh-MYD (InvivoGen, Toulouse, France) for 6 h (37 °C, 95% oxygen, 5% CO₂), after which they were stimulated for 24 h with

Table 2
Cell densities and agonists used in the different reporter cell line assays.

Cell line	Cell density (cell/mL)	Agonist (positive control)	Agonist concentration
THP1-MD2-CD14	5×10^5	LPS-EK	10 ng/mL
HEK-Blue human TLR2	2.8×10^5	Standard lipopolysaccharide from <i>E. coli</i> K12	10 ng/mL
HEK-Blue human TLR3	2.8×10^5	FSL-1 lipopeptide	10 ng/mL
HEK-Blue human TLR4	1.4×10^5	Poly (I:C) HMW Poly (I:C) High Molecular Weight	5 µg/mL
HEK-Blue human TLR5	1.4×10^5	LPS-EK Standard lipopolysaccharide from <i>E. coli</i> K12	10 ng/mL
HEK-Blue human TLR7	2.2×10^5	RecFLA-ST Recombinant flagellin from <i>Salmonella typhimurium</i>	10 ng/mL
HEK-Blue human TLR8	2.2×10^5	CL264 Adenine analog ssRNA40/LyoVec	5 µg/mL
HEK-Blue human TLR9	4.5×10^5	Single stranded RNA ODN 2006 Class B CpG oligonucleotide	0.25 µM

0.5, 1, and 2 mg/mL HMOs or a relevant ligand as a positive control (Table 2). Unstimulated cells were used as negative control. To detect the TLR activation in cells, after 24 h the culture supernatant was mixed with Quanti-Blue detection medium in a ratio of 1:10 in a new plate and incubated for 1 h at 37 °C and 5% CO₂. Absorbance (650 nm) was measured using a Benchmark Plus Microplate Reader using Microplate Manager version 5.2.1 for data acquisition. The data for each sample was plotted as the fold-change compared to the negative control, which was well with unstimulated cells. The assays were performed with three technical replications and each experiment was repeated five times.

2.4. HEK-Blue reporter cell stimulation and Quanti-Blue assay

To assess the activation of individual TLR signaling, HEK-Blue cells were detached from the bottom flask by tapping the flask, after which the cells were centrifuged and resuspended according to the manufacturer's protocol (Table 2). After that, cells were seeded at different cell densities (Table 2) in a flat bottom 96 wells plate at 100 µL per well. The cells were incubated for 24 h (37°C, 95% oxygen, 5% CO₂) with 0.5, 1, and 2 mg/mL HMOs, LNT2 was added to the culture medium after sterile filtration. For each HEK cell line, a corresponding TLR agonist was used as a positive control (Table 2), and the medium was used as a negative control. To exclude possible effects of endotoxin on TLR4 activation, 100 µg/mL polymyxin B was added to capture any LPS present in the 2'-FL, 6'-SL, and LNT2 solutions, as LAL assay detected endotoxin in these three HMOs. After 24 h of incubation, the Quanti-Blue assay was conducted as described above. Data for each sample were plotted as the fold-change compared to the negative control, which was unstimulated cells. The negative controls were set at 1.

Besides activating TLRs, HMOs might also inhibit ligand-induced TLR activation (Sahasrabudhe, Schols, Faas, & de Vos, 2016). Inhibition of the individual TLR was studied by comparing the NF-κB activation of respective TLRs agonists (Table 2) with the cells treated with TLR agonist and sample together. To assess inhibition of the TLR signaling by different concentrations of HMOs, HEK cells were resuspended and seeded in the same way as described above for the activation assays. Then, cells were stimulated with the appropriate TLR ligand (Table 2), together with 0.5, 1, and 2 mg/mL HMOs for 24 h (37 °C, 95% oxygen, 5% CO₂). The TLR ligand alone served as a positive control. After incubation, SEAP activity was performed in the same way as described above (Chanput et al., 2014). Data for each sample were plotted as the fold-change compared to the positive control. The positive controls were set at 1.

The activation and inhibition assays were performed with three technical replicates and each experiment was repeated five times.

2.5. THP1 monocytes cell culture

THP1 monocytes (ATCC TIB-202, Rockville, MD, USA), derived from an acute monocytic leukemia, were maintained in RPMI 1640 medium (Lonza, Verviers, Belgium). Cell culture medium was supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine (Lonza, Verviers, Belgium), 1 mM sodium pyruvate (Lonza, Verviers, Belgium), 0.05 mM 2-mercaptoethanol (Scharlau, Barcelona, Spain), 60 µg/mL gentamicin sulfate (Lonza, Verviers, Belgium), 2.2 µg/mL amphotericin B solubilized (Sigma-Aldrich, St. Louis, MO USA). Cells were incubated at 37 °C with 5% CO₂. The medium was changed every 2 to 3 days, and cells were subcultured when the cell concentration reached 8×10^5 cells/mL.

2.6. THP1 monocytes differentiation to macrophages and stimulation

THP1 monocytes were differentiated into THP1 macrophages as previously described (Ren et al., 2016). Briefly, THP1 monocytes (5×10^5 cells/well, in 0.5 mL) were differentiated for 48 h with 100 ng/mL Phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich) in a 48 wells plate (Corning, New York, USA). After that, PMA was removed and cells were washed twice with fresh culture medium and cultured for another 24 h. Next, the culture medium was discarded and cells were incubated with either 10 µM of the NF-κB inhibitor celastrol (InvivoGen, Toulouse, France) or culture medium for 30 min. Subsequently, fresh medium containing 0.5, 1, and 2 mg/mL HMOs samples or 1 µg/mL LPS (positive control) were added to the cells. Unstimulated cells served as negative control. After 24 h of incubation, the secretion of a proinflammatory (TNFα) and anti-inflammatory cytokine (IL-10) was measured in the supernatant by ELISA (R&D SYSTEM, Minneapolis, MN, USA) according to the manufacturer's protocol.

The stimulation assays were performed with two technical replicates and each experiment was repeated five times.

2.7. Statistical analysis

The results were analyzed using GraphPad Prism. The distribution of the data was tested by using the Kolmogorov-Smirnov test. Values are expressed as mean ± standard deviation (SD). Statistical comparisons of parametric distributed data were performed using unpaired t-tests, one-way ANOVA with Dunn's multiple comparison tests or two-way ANOVA for group analysis. Non-parametric distributed data was assessed using the Kruskal-Wallis test followed by the Dunn's test. $p < 0.05$ was considered as statistically significant ($\hat{p} < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

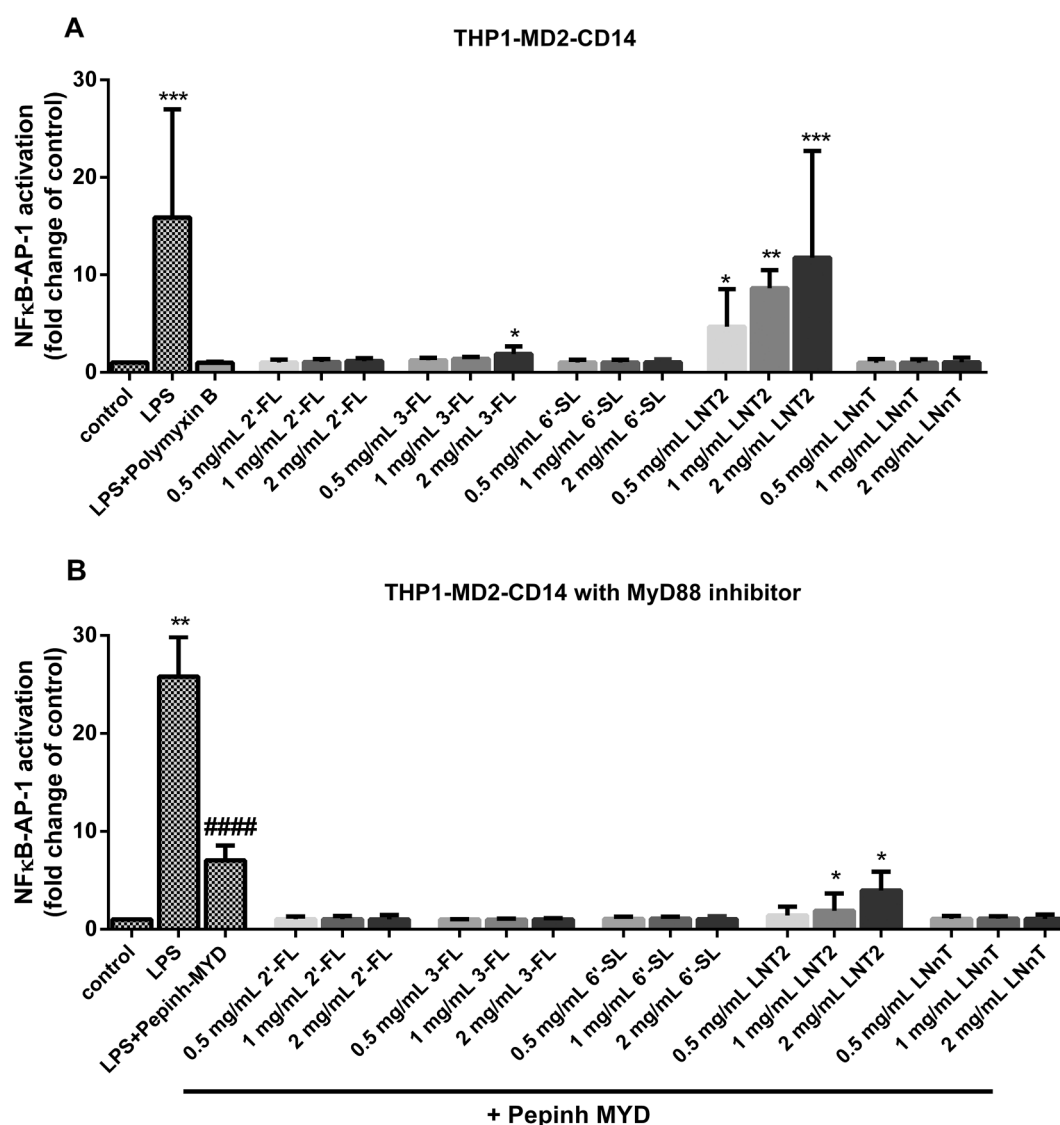


Fig. 1. NF-κB/AP-1 activation in THP1-MD2-CD14 reporter cell and THP1-MD2-CD14 reporter cell with MyD88 inhibitor Pepinh-MYD after stimulation with HMOs. 2'-FL, 3-FL, 6'-SL, LNT2, and LNnT were tested at 0.5, 1, and 2 mg/mL, culture medium served as negative control. A: THP1-MD2-CD14 reporter cell; B: THP1-MD2-CD14 reporter cells with MyD88 inhibitor Pepinh-MYD. Data are presented as mean \pm SD. Significant differences compared to the negative control were determined by using Kruskal-Wallis test followed by the Dunn's test and indicated by #, * p < 0.05; ##, ** p < 0.01; ###, *** p < 0.001; ####, **** p < 0.0001(* vs control; # vs LPS).

3. Results

3.1. Induction of TLR and MyD88-dependent activation in THP1-MD2-CD14 cells by 3-FL and LNT2

To determine whether HMOs induce TLR signaling through the MyD88 pathway, first THP1-MD2-CD14 cells were stimulated with the different HMOs. By comparing the data with stimulated THP1-MD2-CD14 cells in which MyD88 signaling is blocked we determined TLR dependency.

HMOs and its acid hydrolysate LNT2 were tested at a concentration of 0.5, 1, and 2 mg/mL. As shown in Fig. 1A, 2'-FL had no activating effect on NF-κB in THP1-MD2-CD14 cells. The HMO 3-FL only had a statistically significant effect at a concentration of 2 mg/mL (p < 0.05). 6'-SL did not show NF-κB activating effects. For LNT2, 0.5, 1, and 2 mg/mL all showed significantly NF-κB activating effects (0.5 mg/mL, p < 0.05; 1 mg/mL, p < 0.01; 2 mg/mL, p < 0.001). The effects were dose dependent. LNnT had no effect on NF-κB activation.

To determine TLR dependency, THP1-MD2-CD14 cells were incubated with HMOs after inhibition of MyD88 with the MyD88 inhibitor Pepinh-MYD. The efficacy of the MyD88 inhibitor Pepinh-MYD was confirmed on LPS stimulated THP1-MD2-CD14 cells, the effect of LPS was significantly inhibited by Pepinh-MYD (p < 0.0001, Fig. 1B). As shown in Fig. 1B, MyD88 inhibition blocked 3-FL activation demonstrating TLR dependency of this activation. The LNT2 induced activation was partially TLR dependent as some activation was still observed after MyD88 inhibition. Inhibition was dependent on the LNT2 concentration. At 0.5 mg/mL LNT2, the TLR activation was completely blocked (Fig. 1B), at 1 and 2 mg/mL, the response was strongly but not completely blocked (Fig. 1B). Although the remaining activations of 1 and 2 mg/mL LNT2 were still statistically significantly higher than medium control, the response was 6.4 fold and 10.4 fold decreased, demonstrating the response is mainly but not completely TLR dependent (1 mg/mL LNT2, p < 0.001; 2 mg/mL LNT2, p < 0.01).

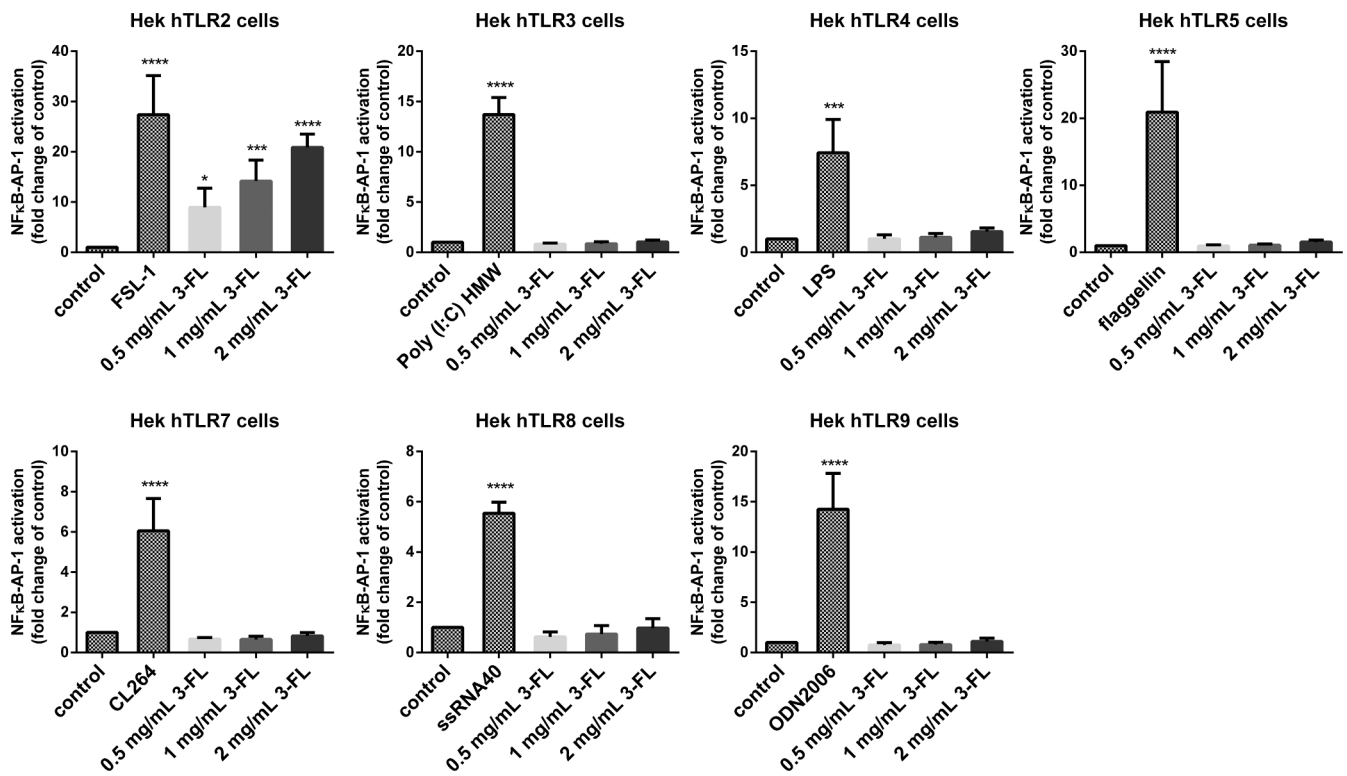


Fig. 2. Activating effects of 3-FL on HEK reporter cells carrying individual TLRs. Cells were incubated with 3-FL at 0.5, 1, 2 mg/mL, culture medium served as negative control. Data are presented as mean \pm SD. Significant differences compared to the negative control were determined by using one-way ANOVA with Dunn's multiple comparison tests and indicated by * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$) or by **** ($p < 0.0001$).

3.2. HMO's effects on individual TLRs

The results from the THP1-MD2-CD14 cell studies showed that 3FL and LNT2 can activate NF- κ B through TLR pathways. In order to investigate which specific TLRs are involved, HEK cells expressing TLR2, TLR3, TLR4, TLR5, TLR7, TLR8, and TLR9 were used. We include all four HMOs and its acid hydrolysate LNT2 and test for both activating and inhibiting effects to exclude any possible effects of the individual HMOs on TLR.

To study possible activating effects, 0.5, 1, or 2 mg/mL of 2'-FL, 3-FL, 6'-SL, LNT2, and LNT were added to the cells. The results of the activating effects of 2'-FL, 6'-SL, and LNT on the individual TLRs are shown in Figs. S2, S3, and S4, respectively. The results corroborate the observation in THP1 cells and show that these HMOs had no activating effects on any of the TLRs tested. Only 3-FL and LNT2 had activation effects which results are shown in Figs. 2 and 3. The HMO 3-FL only activated TLR2 (Fig. 2). The activating effects were dose dependent. At a concentration of 0.5, 1, and 2 mg/mL the 3-FL induced an 8.9 ($p < 0.05$), 14.1 ($p < 0.001$), and 20.9 ($p < 0.0001$) fold increase of NF- κ B activation respectively compared to control.

LNT2 statistically significantly activated all the TLRs in a dose-dependent way (Fig. 3). LNT2 at 0.5 mg/mL activated only TLR2, 5, and 9 (TLR2 and 5: $p < 0.0001$; TLR9: $p < 0.05$); LNT2 at 1 mg/mL activated TLR2, 3, 5, 8, 9 (TLR2, 5, and 9: $p < 0.001$; TLR3, 8: $p < 0.05$); LNT2 at 2 mg/mL activated all the TLRs (TLR2, 5, and 9: $p < 0.001$; TLR3, 4, 7, and 8: $p < 0.01$). It showed a different activation pattern on different TLRs, but for all the TLRs, the activation effect was increasing with increasing concentrations.

To exclude this we performed additional controls but also our data indicate it can not be explained by endotoxin only. First, we added the LNT2 to the culture medium after sterile 0.2 μ m filtration which removes bacteria and sterilize the solutions. Subsequently the LAL assay indicate that the 2 mg/mL LNT2 showed high endotoxin levels (1.7 EU/mL) while 0.5 mg/mL 0.2 μ m filtration LNT2 contained very low

endotoxin levels (< 0.1 EU/mL) (Fig. S1). This 0.5 mg/mL LNT2 with virtually no endotoxin still induced activation on TLR2, 5, and 9 indicating the LNT2 is responsible for TLR activation. However to further exclude that the TLR4 activation of the LNT2 was caused by possible contamination with LPS, we added 100 μ g/mL polymyxin B to the LNT2 to capture any LPS present in the samples (Fig. S5). The effectivity of polymyxin B was confirmed on TLR4 reporter cells, as a complete blockade of the LPS signal was observed (Fig. S5A). Polymyxin B reduced the LNT2 induced TLR4 activation but the remaining activation was still profound and statistically significantly higher than medium control (Fig. S5B). Also as TLR2 and 4, i.e. the predominant receptors for endotoxins, were not more strongly activated than other receptors we conclude that the observed effects were predominantly caused by the LNT2 itself.

We also investigated such ability of 2'-FL, 3-FL, 6'-SL, LNT2, and LNT. This was done by incubating HEK-TLR cells with 0.5, 1, and 2 mg/mL HMOs and relevant TLR agonist. We found that 2'-FL, 6'-SL and LNT had some inhibiting effects on TLR signaling as will be specified in more detail below. TLR2, 3, 4, and 9 was not inhibited by any of the tested HMOs. The full range of outcomes is shown in Fig. S6. Fig. 4 presents only the TLRs which were inhibited by HMOs. As shown in Fig. 4A, 2'-FL, 3-FL, 6'-SL, and LNT suppressed TLR5 activation only at the lowest concentration HMOs tested (0.5 mg/mL, all $p < 0.05$). No inhibition was observed at concentrations of 1 and 2 mg/mL. Such an inverse dose dependent effect was observed for TLR7 suppression as well. Both at 0.5 mg/mL of 2'-FL ($p < 0.05$), 3-FL ($p < 0.01$), 6'-SL ($p < 0.01$) and 1 mg/mL of 2'-FL ($p < 0.05$), 3-FL ($p < 0.05$), 6'-SL ($p < 0.01$), and LNT ($p < 0.05$) inhibition of CL264 induced TLR7 activation was observed, while none of the HMOs showed significant effects on TLR7 inhibition at a concentration of 2 mg/mL (Fig. 4B). For TLR8 inhibition, different results were obtained, the inhibition effects on TLR8 were enhanced with increasing concentrations. Only 1 and 2 mg/mL 3-FL (both $p < 0.01$) showed significant inhibition of TLR8 activation, while 0.5 mg/mL 3-FL showed a trend to suppress TLR8

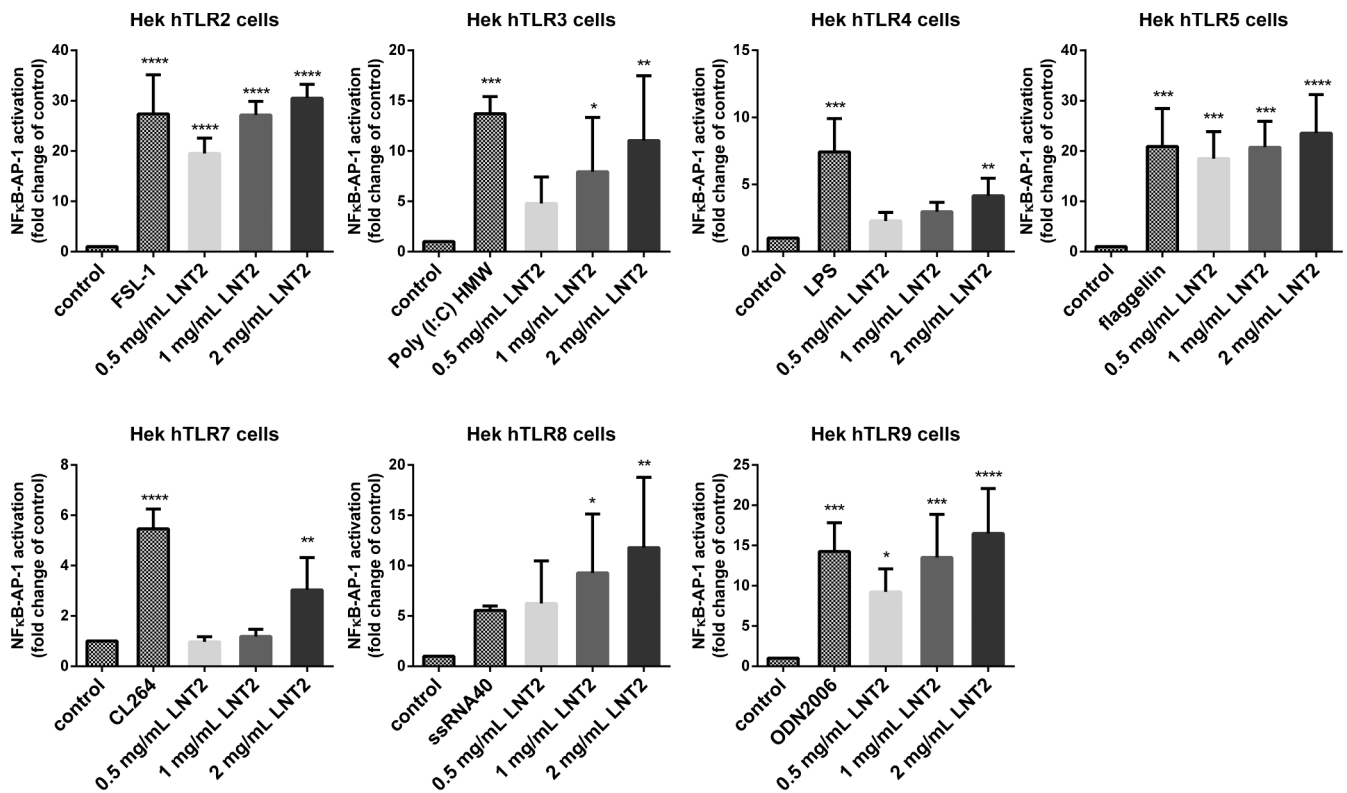


Fig. 3. Activation effects of LNT2 on HEK reporter cells carrying individual TLRs. Cells were incubated with LNT2 at 0.5, 1, 2 mg/mL, culture medium served as negative control. Data are presented as mean \pm SD. Significant differences compared to the negative control were determined by using one-way ANOVA with Dunn's multiple comparison tests and indicated by * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$) or by **** ($p < 0.0001$).

activation to 82.2% ($p = 0.07$, Fig. 4C). Also, we observed that 6'-SL and LNT2 showed significant enhancing effects on TLR8 activation but only at the highest concentration of 2 mg/mL ($p < 0.01$). Interestingly, 6'-SL did not inhibit but augmented TLR8 activation in the presence of the agonist (ssRNA40) at a concentration of 2 mg/mL while it had no activation effect as a single molecule on TLR8.

3.3. Induction of cytokine production in THP1 macrophages by LNT2

The above-mentioned activation and inhibition of TLRs by HMOs and its acid hydrolysate LNT2 promoted us to test whether HMOs have immunomodulatory effects on TLR carrying cells such as macrophages. To this end, PMA-differentiated THP1 macrophages were stimulated with 0.5, 1, and 2 mg/mL HMOs for 24 h after which the concentrations of the pro-inflammatory cytokine TNF α and the regulatory cytokine IL-10 in the supernatant were measured.

As shown in Fig. 5, only LNT2 could significantly increase cytokine production of TNF α and IL-10, and the effects were dose dependent. LNT2 was able to induce high TNF α production in all tested concentrations (0.5 and 1 mg/mL: $p < 0.001$; 2 mg/mL: $p < 0.0001$), while for IL-10, only 1 and 2 mg/mL LNT2 could significantly increase IL-10 production (1 mg/mL, $p < 0.05$; 2 mg/mL, $p < 0.01$). Incubation with 0.5 mg/mL LNT2 was not able to increase IL-10 production significantly, but it did show a trend to inducing enhanced IL-10 production by the macrophages ($p = 0.069$).

3.4. Cytokine expression was NF- κ B dependent in THP1 macrophages

In order to confirm the NF- κ B dependency of cytokine expression induced by LNT2, PMA-differentiated THP1 macrophages were stimulated with 0.5, 1, and 2 mg/mL LNT2 in the presence or absence of the NF- κ B inhibitor celestrol. As shown in Fig. 6, incubation with celestrol inhibited production of TNF α and IL-10 induced by LNT2. TNF α

produced by the THP1 macrophages after incubating with 0.5 mg/mL ($p < 0.05$), 1 mg/mL ($p < 0.001$) and 2 mg/mL ($p < 0.01$) LNT2 was significantly decreased by celestrol. IL-10 production induced by 1 and 2 mg/mL LNT2 ($p < 0.05$) was also inhibited by celestrol. Celestrol also showed a trend of decreasing the IL-10 production induced by 0.5 mg/mL LNT2 ($p = 0.075$). Overall, the data above showed the TNF α and IL-10 production induced by LNT2 was NF- κ B dependent.

4. Discussion

The immune effects of HMOs through TLRs have been previously described *in vitro* (Asakuma et al., 2010; He et al., 2014; Thomas et al., 2014), and *in vivo* (Kurakevich et al., 2013), however, the effects of different HMOs on individual TLRs are to the best of our knowledge not studied before. Here, we show that the immunomodulatory effects via TLRs of 2'-FL, 3-FL, 6'-SL, LNT2, and LNnT, is HMO structure-dependent. While 3-FL and LNT2 had TLR stimulating effects, the 2'-FL, 6'-SL, and LNnT were inhibiting TLR5 and 7, besides that, 3-FL also showed inhibition effects on TLR5, 7, and 8.

3-FL showed direct stimulatory effects on TLR2 in a dose-dependent way. Notably, 2'-FL and 3-FL have approximately the same molecular composition. Sharing the same molecular composition, all carrying lactose at their reducing end. 2'-FL and 3-FL only differ in the attachment position of L-fucose (Fuc) residues on the lactose core region (Table 1). This difference might, however, be responsible for the difference in binding capacity to TLRs. TLR2 can form a specific binding pocket of equine TLR2/1 and TLR2/6 heterodimers (Botos, Segal, & Davies, 2011; Irvine, Hopkins, Gangloff, & Bryant, 2013). Differences in the structural makeup of Fuc on the backbone between 2'-FL and 3-FL might be responsible for the different effects on TLR2 between the two molecules and might make that only 3-FL can bind to the ligand position and activate TLR2. It has been shown for other carbohydrates that minor differences in structural makeup have a significant impact on the

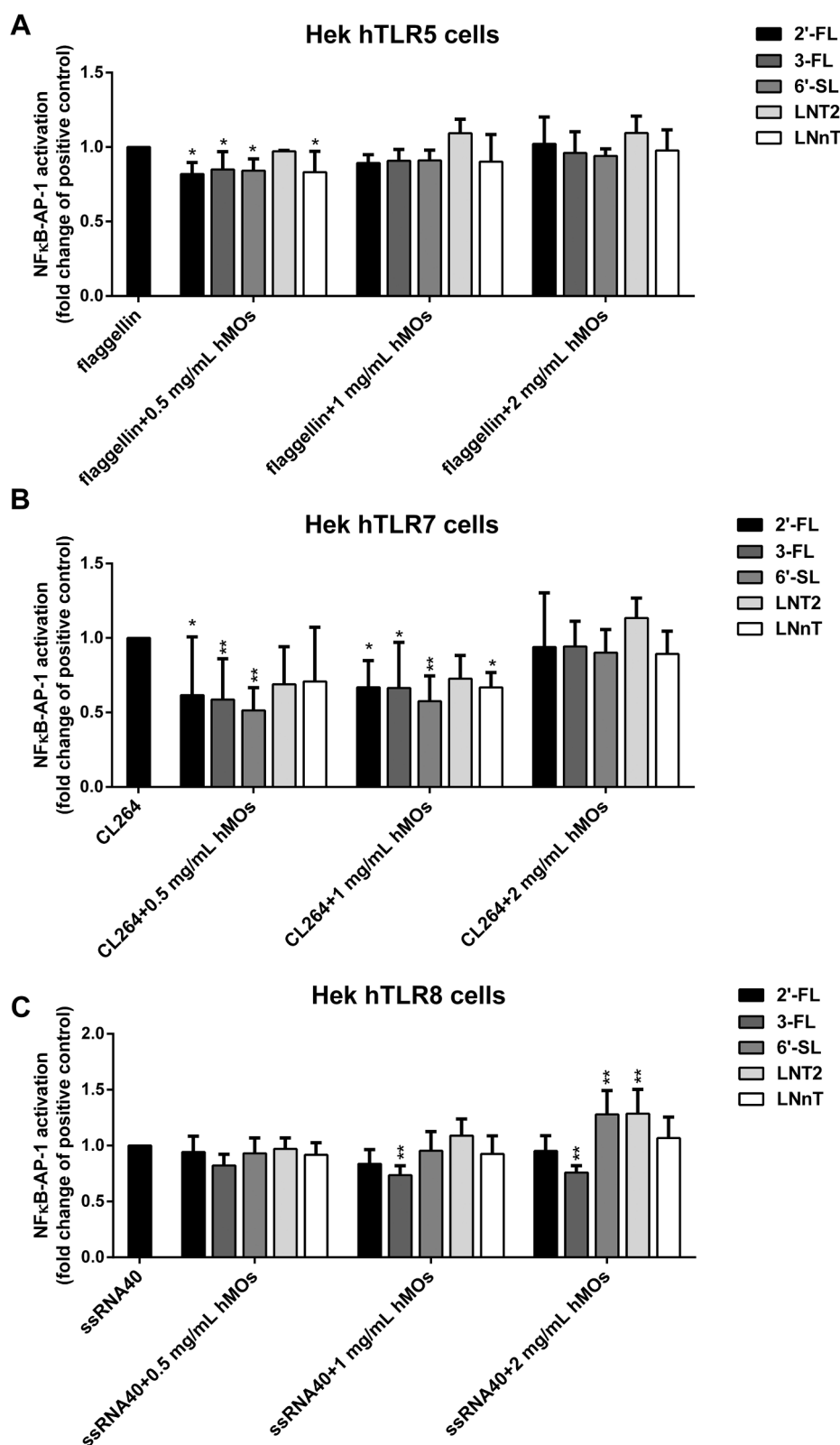


Fig. 4. Inhibitory effects of HMOs on (A) HEK-Blue hTLR5, (B) HEK-Blue hTLR7, and (C) HEK-Blue hTLR8 cells. Cells were incubated with 2'-FL, 3-FL, 6'-SL, LNT2, and LNnT at 0.5, 1, 2 mg/mL together with its relevant agonist. Data are presented as mean \pm SD. Significant differences compared to the control (relevant agonist) were determined by using two-way ANOVA and indicated by * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$) or by **** ($p < 0.0001$).

binding capacity to especially TLR2 (Sahasrabudhe et al., 2018).

Despite its TLR2 activating effect, direct stimulation of 3-FL on THP1 macrophages did not induce any change in cytokine production. It was recently shown that the final effects of food ingredients on NF- κ B

regulation in THP1 macrophages dependent on the sum of activating and inhibiting effects on TLRs (Kiewiet et al., 2017; Lépine & de Vos, 2018; Sahasrabudhe et al., 2018). In addition to activating TLR2, we found that 3-FL inhibited TLR 5, 7, and 8 (Fig. 7A). As the net effects of

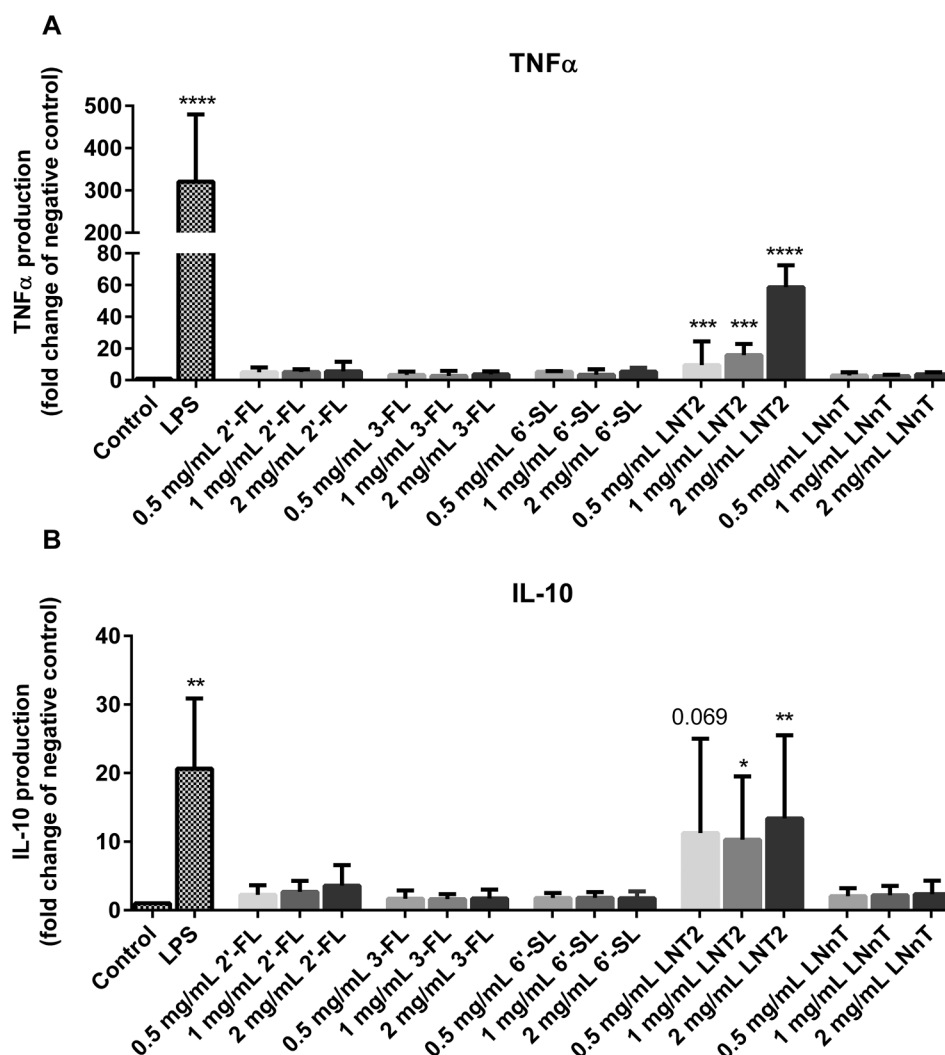


Fig. 5. (A) TNF α and (B) IL-10 production by THP1 macrophages stimulated with 0.5, 1, and 2 mg/mL HMOs. PMA-stimulated THP1 macrophages were treated with 2'-FL, 3-FL, 6'-SL, LNT2, and LNT2 at 0.5, 1, 2 mg/mL or 1 μ g/mL LPS (positive control) for 24 h. Untreated THP1 macrophages served as negative control. Data are presented as mean \pm SD. Significant differences compared to the negative control were determined by using Kruskal-Wallis test followed by the Dunn's test and indicated by * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$) or by **** ($p < 0.0001$).

immune activation depend on the sum of activation and inhibition of TLRs, the final effects of this combined effects on TLRs are probably lack of activation of the macrophages. This is corroborated by the observation on THP1 cells (Fig. 1) in which modest overall stimulation of THP1 cells was observed by 3-FL. Only at a concentration of 2 mg/mL, we observed a modest activation of 1.89 times fold-change of NF- κ B.

Stimulating effects of LNT2 were observed on all TLRs tested (Fig. 7B). To the best of our knowledge, direct activation effects of TLRs by LNT2 have not been reported before. It might be argued that the strong and broad LNT2 TLR activating effect was caused by endotoxins in the samples. Our data indicate this activation cannot be solely explained by the positive signal in the LAL assay at 2 mg/mL. At 0.5 mg/mL LNT2, the LNT2 had very low endotoxin levels (< 0.1 EU/mL) (Fig. S1) but still demonstrated TLR activation. Another experiment that made us conclude that LNT2 itself is partly responsible for TLR4 activation is that polymyxin B, a potent LPS neutralizer reduced TLR4 activation by LNT2 but LNT2 still showed TLR activation (Fig. S5B). As the difference in the LAL assay did not reflect the only fourfold difference in concentration, we suspect that the 2 mg/mL endotoxin value might be a false positive signal. It has been reported before that some LAL reactive glucans might result in the false positive signals in the LAL assay (Roslansky & Novitsky, 1991), the large difference in LAL assay outcome between the 0.5 mg/mL and 2 mg/mL might suggest a false

positive signal in our study as well.

The activating effects of LNT2 might be explained by its structural composition that allows for interaction with N-terminal ectodomains (ECDs) that are present on all TLRs. The ECDs are composed of approximately 16–28 of a motif known as the leucine-rich repeats (LRR). Each LRR consists of 20–30 amino acids with the conserved motif “LxxLxLxxN” (Botos et al., 2011; Jin & Lee, 2008; Kumar, Kawai, & Akira, 2009). All LRRs can form a loop structure, beginning with an extended stretch that contains three residues in the β strand configuration (Bella, Hindle, McEwan, & Lovell, 2008). Different from other tested samples, LNT2 has the above mentioned N-acetylglucosamine (GlcNAc) end (Table 1), which plays a variety of roles on the cell surface of bacteria (Camacho et al., 2017; Konopka, 2012). This GlcNAc might interact with LRR, which is the conserved part of TLRs, resulting in the activation of all TLRs we tested. The activating effects on individual TLRs were different, but the effect is enhanced as the concentration increases (Fig. 3). This suggests that the TLR interaction effects are dose-dependent. The effects of LNT2 on direct immune stimulation were confirmed by exposing THP1 macrophage to LNT2 which resulted in enhanced TNF α , and IL-10 production through the NF- κ B pathway (Fig. 7B).

We did not detect activating effects of 6'-SL on TLR2 and TLR4, which differs from the observations of Asakuma (Asakuma et al., 2010).

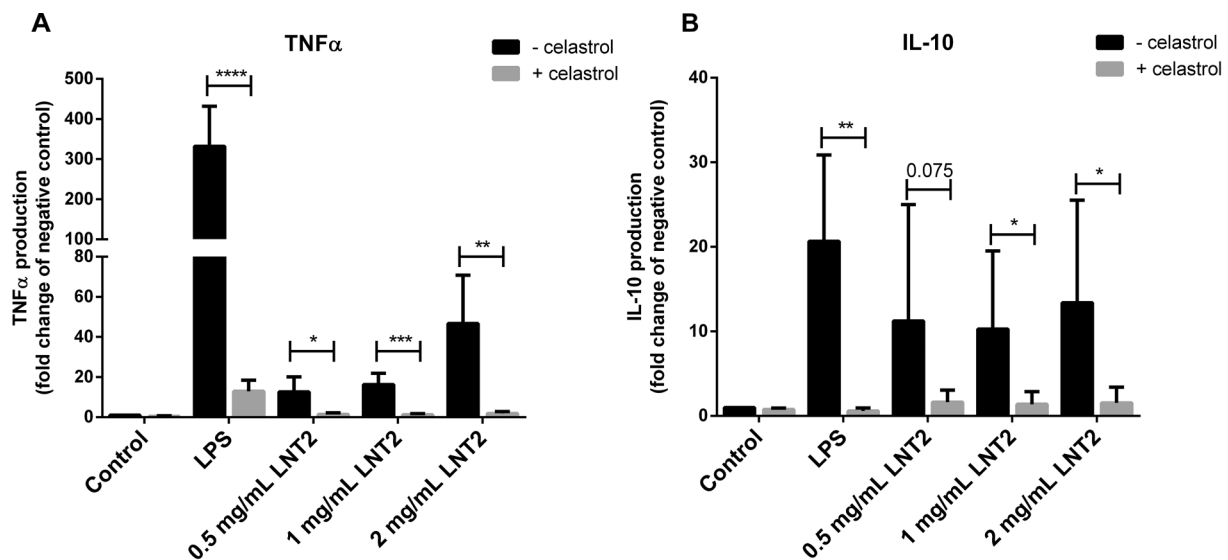


Fig. 6. (A) TNF α and (B) IL-10 production by THP1 macrophages stimulated with 0.5, 1, and 2 mg/mL LNT2 in the presence or absence of celastrol. PMA-stimulated THP1 macrophages were treated with LNT2 at 0.5, 1, 2 mg/mL or 1 μ g/mL LPS (positive control) in the presence or absence of NF- κ B inhibitors celastrol for 24 h. Untreated THP1 macrophages served as negative control. Data are presented as mean \pm SD. Significant differences were determined by using unpaired *t*-test and indicated by * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$) or by **** ($p < 0.0001$).

Asakuma has suggested that 6'-SL has a direct effect on colonic epithelial HT-29 cells and induces mRNA expression of TLR2 and TLR4 (Asakuma et al., 2010). TLR4 and to a lesser extent TLR2 are receptors that are sensitive for endotoxins present in food components. It cannot be excluded that possible contamination in the 6'-SL in the Asakuma study has been responsible for the TLR2 and TLR4 activating effects and explains the discrepancy between our findings and that of the Asakuma study. In our study, we applied virtually endotoxin free 6'-SL (Figure S1), which made us believe we can safely conclude that 6'-SL has no TLR2 and TLR4 activating effects.

As it has been shown that TLRs can not only be activated but also be inhibited by carbohydrates (Figuerola-Lozano et al., 2018; Kiewiet et al., 2017; Sahasrabudhe et al., 2018), the inhibiting effects were tested as a potential mechanism for immunomodulation. We observed that 3-FL not only showed activation effects on TLR2 but also had inhibiting effects on TLR 5, 7, and 8. Also, 2'-FL, 6'-SL, and LNT2 showed TLR inhibiting effects while no activating effects were found with these HMOs (Fig. 7C). A few studies demonstrate that inhibition of TLR 5, 7, and 8 might be instrumental in managing inflammatory and autoimmune diseases (Blohmke et al., 2008; Kuznik et al., 2011). As 2'-FL, 3-FL, 6'-SL, and LNT2 have these inhibitory effects, they might be therapeutically applicable. To our surprise, the results indicated that the inhibition effects on TLR5 and 7 were inversely dose-dependent. Low concentrations of HMOs inhibited the activation effects on TLR5 and 7 induced by agonist, whereas high concentrations had no effects. The inverse dose-dependent manner had been reported to be present for other antagonists (Palmon et al., 2000; Tardy et al., 2015). For example, low concentrations of basic fibroblast growth factor (bFGF) enhanced collagen type I and depressed collagenase-1 expression, whereas high bFGF concentrations had no inhibiting effects (Palmon et al., 2000). To the best of our knowledge, this inversely dose-dependent inhibition effects on TLRs have not been reported before. These results suggested that 2'-FL, 3-FL, 6'-SL, and LNT2 might be important regulators of the TLR dependent NF- κ B pathway.

Notably, 2 mg/mL 6'-SL had a synergistic effect on ssRNA40 induced TLR8 activation while this HMO had no effect as a single molecule on TLR8. TLR8 activation in contrast to activation of other TLRs is a multistep process. TLR8 activation first requires the formation of an apo TLR8 dimer after a proteolytic cleavage that subsequently induces a conformational change of TLR8 upon ligand binding (Geyer, Pelka, &

Latz, 2015; Tanji et al., 2015, 2016; Yin, Fu, Li, & Wu, 2015). Upon binding of ssRNA to the TLR8 binding site, an activated dimer is formed with the uridine part of ssRNA in the receptor (Botos et al., 2011; Tanji et al., 2015). This changes the ligand binding site and the distance between the C-termini that might be responsible for binding of other ligands such as 6'-SL (Geyer et al., 2015; Tanji et al., 2015). The C termini in the agonist-bound TLR8 dimer are much closer to each other than those in the inactivated-TLR8 dimer. The measured distances between the C termini in activated TLR8 was ~ 30 Å which is smaller than the ~ 53 Å in the inactivated TLR8 dimers (Yin et al., 2015). This difference in the distance might explain the difference in binding and activating capacity of 6'-SL between inactivated the form of TLR8 and the activated TLR8 (Fig. 7D).

5. Conclusion

We demonstrate that the immunomodulatory effects of HMOs are highly structure dependent. Different structures of HMOs showed different activation and inhibition effects on TLR signaling pathways. Our data indicate that some specific HMOs, such as LNT2, are able to stimulate immune activation. We also identified that TLR 5, 7, and 8, which are involved in many pathologies, such as cystic fibrosis lung disease and systemic lupus erythematosus (Blohmke et al., 2008; Kuznik et al., 2011), were inhibited by some specific HMOs. Understanding how and which HMO modulate immunity, contributes to the future design of HMO containing products with predictable beneficial effects in specific target groups. Overall, our data not only contribute to a better understanding of how HMOs can have immunomodulatory effects, it also provides a new way to manage diseases, like systemic lupus erythematosus and rheumatoid arthritis (Karlsson, Sun, Rao, Venable, & Thurmond, 2008), with nutritional supply.

6. Ethics statements

No human subject and animal experiments in the research.

Author contributions

L.C., M.B.G.K., and P.d.V conceived and designed the experiments. L.C. performed the experiments. L.C. analyzed data. A.G. and A.N.

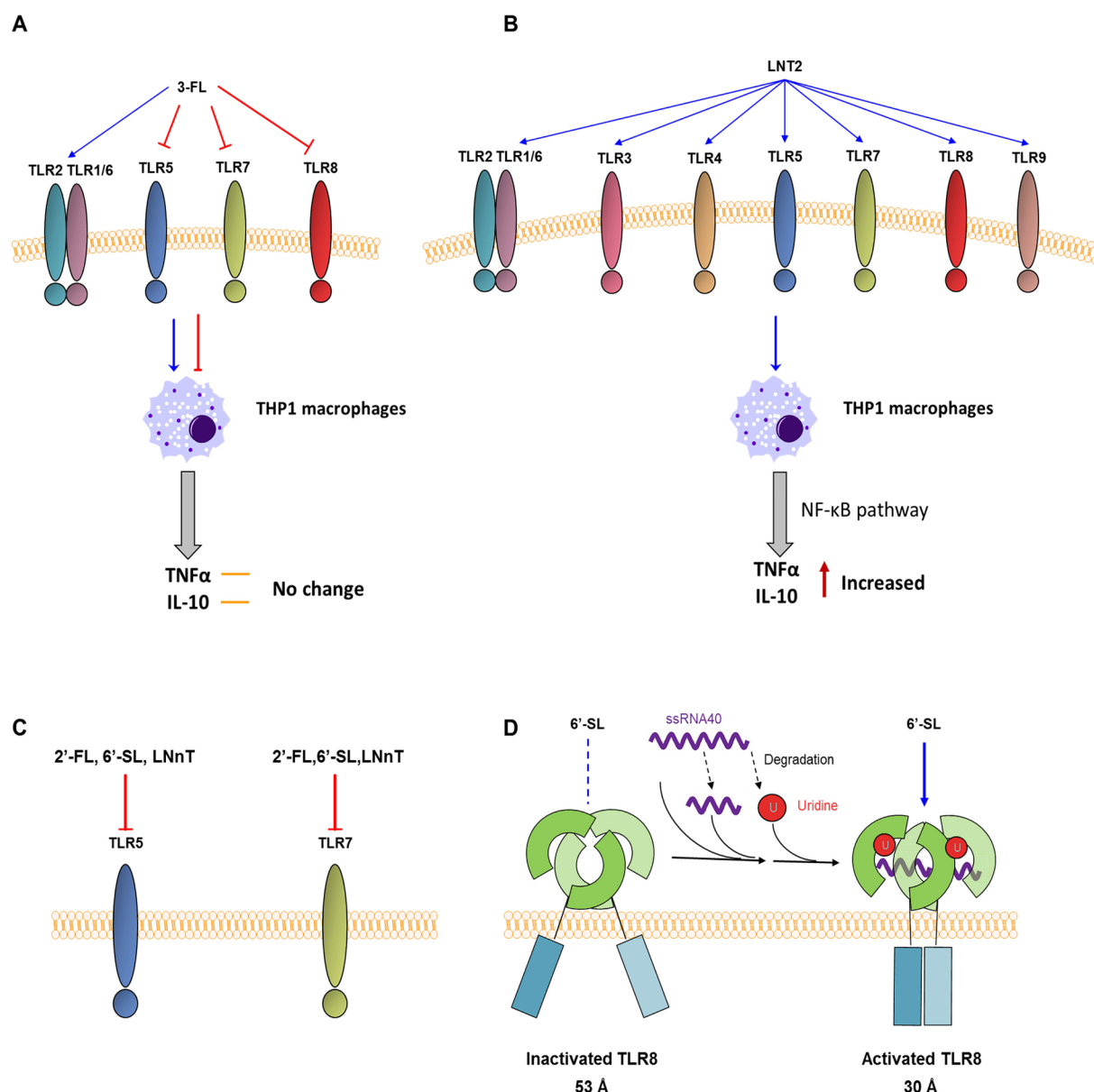


Fig. 7. Schematic representation of the immunomodulatory effects of tested HMOs. A–C: Immune regulation effects of HMOs through TLR signal pathway; D: Proposed mechanism of activation effect of 6'-SL on ssRNA40 activated TLR8. —▶: activation effect; —⊥: inhibition effect; - - - - -: no significant effect.

provided the tested samples and joined the discussion. L.C., M.B.G.K., and P.d.V. wrote the paper.

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Declaration of Competing Interest

The authors have declared no conflict of interest.

Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jff.2019.05.023>.

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